NEW OLIGOMERIC CONJUGATES LIABLE TO TRANSFER BIOLOGICAL MOLECULES INTO CELLS

The invention relates to new oligomeric conjugates liable to favor the transfer of biological molecules such as oligonucleotides, peptides and oligosides into cells.

The introduction of such molecules into cells is of great therapeutical interest.

Antisense oligonucleotides (ODN) and triplex forming oligonucleotides (TFO) are examples of attractive putative drugs in inhibiting or regulating gene expression in tumor cells and virus-infected cells.

Peptides from tumors and viruses are also attractive molecules in stimulating or elicitating a cell defense involving cytotoxic T lymphocytes against tumor and viruses after presentation by antigen presenting cells (APCs) such as dentrictic cells, macrophages and B cells.

But to be effective these molecules must reach their target in the right intracellular compartments which are either the cytosol or the nucleus.

Until now, studies of the intracellular location of oligonucleotides point out that in the majority of cells, most of the oligonucleotides are confined inside vesicles once taken up and only a small amount succeeds in reaching their RNA and DNA targets in the cytosol and in the cell nucleus.

Because once in the cytosol, the oligonucleotides penetrate rapidly into the nucleus, the enhancement of the delivery of oligonucleotides into the cytosol upon cell uptake, is expected to increase their biological activity.

Oligonucleotide encapsulation into liposomes increases their delivery into the cytosol but efficiency is drastically reduced in the presence of serum.

Antigenic peptides can bind to MHC Class I molecules and be presented at the cell surface of APCs, upon their processing via proteasomes located in the cytosol.

Usually, peptides are taken up by APCs inside vesicles and delivered to lysosomes where they are either degraded or processed for an MHC Class II molecules presentation. Restricted MHC Class I molecules presentation by APCs requires that peptides must be introduced into the cytosol.

In French Patent n° 9613990 and PCT/FR97/02022, it has been shown that histidylated polylysine complexed with a nucleic acid is a system for cell transfection. The nucleic acid has a 10^6 to 10^8 of molecular weight. Polylysine is substituted at least 10% advantageously from 15% to 35% with molecules inducing membrane destabilization at acidic pH (mainly histidyl residues), and polylysine has a degree of polymerization of 15 to 900, particularly 200.

However, further studies have shown that the above-mentioned complex, which allows the transfection of cells by DNA, does not allow the transfer of an ODN (oligonucleotide).

One aim of the invention is to provide new positivly charged oligomeric conjugates enabling the transmembrane passage of water soluble oligomers such as oligonucleotides, peptides and oligosides into the cytosol.

An other aim of the invention is to provide new oligomeric conjugates of substituted oligolysine liable to allow the transfer of oligonucleotides, peptides and oligosides into cells.

One advantage of the invention is that the formation of a complex between new oligomeric conjugates and oligoanions such as oligonucleotides, anionic peptides or anionic oligosides, is not required to allow their transfer into the cytosol and/or the cell nucleus.

Another advantage of the invention is that, although it is not excluded, the transfer of oligoanions such as oligonucleotides, anionic peptides, anionic oligosides (i.e. sulphated, phosphorylated, succinylated or sialytaded oligosides) or a mixture thereof, does not require the formation of electrostatic complexes with the new positively charged oligomeric conjugates.

Another aim of the invention is to provide an *in vitro*, *ex vivo* and *in vivo* transfer process.

Another aim of the invention is to provide defined oligmeric compounds in which the nature of the monomeric compounds can be different from each other.

This has been achieved through the invention.

The invention, in one of its most general definitions, concerns a positively charged oligomeric conjugate containing an oligomer with a polymerization degree (PD) from 5 to 50, preferably 10 to 40 and more preferably 20, formed from monomeric components having free NH_3^+ in a number equal to or higher than 50 % of the polymerization degree,

said oligomer being as follows :

- the free NH_3^+ of the above-mentioned components are substituted in a ratio of at least 50 %, advantageously from 60 % to 95 %, particularly 80 to 90 % (this ratio being determined by nuclear magnetic resonance), by protonable residues in a weak acid medium, leading in such weak acid medium to a destabilization of cellular membranes,

- the above-mentioned protonable residues possess in addition the following properties :

→ they contain a functional group enabling them to be linked to the above-mentioned oligomer,

→ they do not correspond to a recognition signal recognized by a cellular membrane receptor,

→ they can comprise at least one free NH_3^+ group,

- the free NH_3^+ of the above-mentioned monomers can be also substituted by an uncharged residue leading to a reduction of the number of positive charges in comparison to the same oligomeric conjugate, before substitution,

- molecules constituting a recognition signal recognized by a membrane cellular receptor may be present :

→ either by substitution of some of the free NH_3^+ of the above-mentioned monomers,

→ either on some of the uncharged residues leading to a reduction of the number of charges,

→ either on some of the above-mentioned protonable residues leading to a destabilization of the cellular membranes,

→ or by substitution of the free NH_3^+ (if it is present) of the above-mentioned protonable residues leading to a destabilization of the cellular membrane,

provided that :

1) the total number of the non substituted NH_3^+ is of at least 50 % of the polymerization degree,

2) the number of monomers initially carrying free NH_3^+ is substituted in a ratio of at least 50 % of the polymerization degree by residues leading to a destabilization of the cellular membrane.

As it will result from the following, the first proviso corresponds to $m \geq i/2$ and the second proviso corresponds to $u \geq i/2$.

Said oligomeric conjugate of the invention leads to the destabilization of the membranes and allows the transfer of the above-mentioned biomolecules in the cytosol and/or the nucleus of the cells.

According to a quite unexpected effect, the oligomeric conjugates of the invention allow the transfer of ODN into the cytosol and the nucleus, without allowing the transfection of cells by DNA.

The originality of the invention lies in the fact that the oligomer of the invention must be substituted to a level of more than 50 % with molecules inducing a membrane destabilization at acidic pH (amongst such molecules are histidyl residues) because oligomeric conjugates substituted at a level lower than 50 % are cytotoxic (see table 1, hereafter). Moreover, the toxicity of oligomeric conjugates substituted at a level lower than 50 % increases with the number of free NH_3^+ of the unsubstituted lysyl residues.

For instance, for a oligomer of lysine of DP of 20 and carrying histidyl residues leading to the destabilization of the membranes, the above-said conditions can be expressed as follows :

- there must be at least 10 free NH_3^+, which come from the α-NH_3^+ and/or ε -NH_3^+,

- there are at least 10 histidyl residues, and even up to 20 histidyl residues carried by the side chain of the lysine residues which means, in the latter case, that

the minimum amount of free NH_3^+ required, i.e. "at least" 10 free NH_3^+ come only from the α-NH_3^+ functions of the histidyl residues.

The destabilization of membranes means a modification of membranes which leads either to the increase of their permeability with respect to low molecular weight (and possibly high molecular weight) molecules in solution, or the fusion with another membrane.

The membrane permeability can be measured as follows :

Cells are incubated at 37°C for 30 min in DMEM medium without serum in the presence of 0.5 mg/ml fluorescein-labelled dextran (Mw 4000) and in the absence or in the presence of an oligomeric conjugate. Cells are then washed and incubated for 30 min at 37°C in culture medium containing 10% serum. Cells are fixed for 5 min in PBS containing 4 % paraformaldehyde and the cell fluorescence localization is analysed under a fluorescent confocal microscope.

The fusion of membrane can be measured as follows :

The fusion of membrane can be measured by using liposomes according to Struck et al., (Use of resonance energy transfer to membrane fusion. 1981 Biochemistry 20: 4093-4099).

Dioleoylphosphatidylcholine (DOPC) liposomes containing N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) and octtadecylrhodamine (R18) as fluorescent energy transfer donor and acceptor lipid probes, respectively, are mixed with non fluorescent liposomes and incubated in the absence or in the presence of oligomeric conjugates at various pH. Membrane fusion is evidenced by a decrease of the rhodamine fluorescence emission at 585 nm upon excitation at 470 nm, as a consequence of a decrease of the resonance energy transfer between NBD and rhodamine when the average spatial separation induced by membrane fusion increases.

The residues accounting for the destabilization of cellular membranes act through their property of being protonable in a weak acid medium.

The expression "weak acid medium" designates a medium the pH of which is lower than that of plasma or serum, i.e. a pH lower than 7.4.

Said medium can be either the extracellular medium or the lumen of intracellular compartments such as endosomes or lysosomes.

Said medium can be naturally acid or be acidified.

By way of example, the pH of said medium can be in the range of about 5 to about 7, in particular 5.5 to 6.5.

Transfer of the above-mentioned biomolecules into the cytosol and/or the cell nucleus, requires that both the oligomeric conjugate leading to the membrane destabilization and the above-mentioned biomolecules are present in the said medium.

In the present invention, the oligomeric conjugates and the above-mentioned biomolecules can be free or complexed.

Said positively charged oligomeric conjugate of the invention is liable to form a complex with at least one negatively charged oligoanion, the association between the oligoanion and the oligomeric conjugate being electrostatic in nature.

The expression according to which the protonable residues do not correspond to a recognition signal recognized by a cellular membrane receptor means that these residues are not used as ligands.

A molecule or a molecular complex is active as a recognition signal when it can be selectively recognized by a receptor, that is to say plays the role of a ligand, of an agonist, or of an antagonist. By recognition signal recognized by a cellular membrane receptor, one designates a ligand (molecule or molecular complexes) liable to be selectively recognized by said receptor.

According to an advantageous embodiment, the invention relates to a oligomeric conjugate, wherein the protonable residues leading to a destabilization of cellular membranes, present the additional properties :

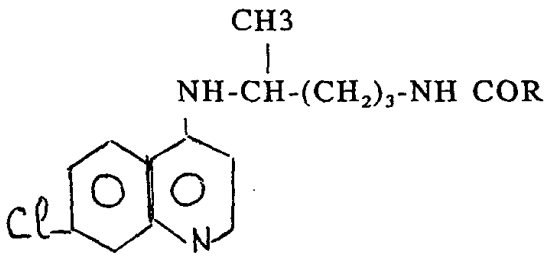
- they are weak bases, the pK of which in aqueous medium is lower than 8, so that a proportion higher than 50 % of these bases linked to a cationic oligomer is not protonated at pH 7.4.

According to another advantageous embodiment, in the oligomeric conjugate of the invention, the protonable residues leading to a destabilization of cellular membranes, present the additional properties :

- they belong to the group of compounds comprising an imidazole ring,

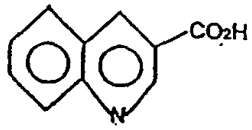
- they belong to the group of quinolins,
- they belong to the group of pterins,
- they belong to the group of pyridins.

An example of quinolin is represented by the following formulae :



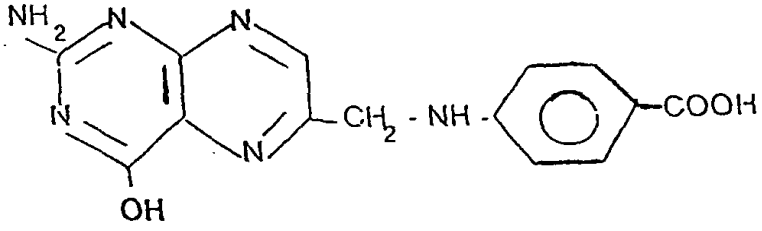
wherein R = $(CH2)_nCO_2H$, in which n varies from 1 to 10, preferably from 1 to 3.

Another example of quinolin is :



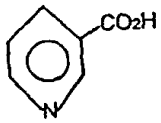
(3-quinoline carboxylic acid)

An example of pterin is represented by the following formula :

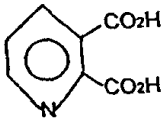


(pteroic acid)

Examples of pyridins are represented by the following formulae :



(nicotinic acid)



(quinolinic acid)

According to an advantageous embodiment, in the oligomeric conjugate of the invention, the protonable residues leading to a destabilization of the cellular membranes are :

- alkylimidazoles in which the alkyl radical comprises from 1 to 10, particularly from 2 to 6 carbon atoms, and in which only one of the nitrogen atoms of the imidazole ring is substituted.

According to an advantageous embodiment, in the oligomeric conjugate of the invention, the protonable residues leading to a destabilization of cellular membranes are chosen from :

histidine, 4-carboxymethyl-imidazole,
3-(1-methyl-imidazol-4yl)-alanine, 3-(3-methyl-imidazol-4yl)-alanine,
2-carboxy-imidazole, histamine, 3-imidazol-4yl)-L-lactic acid,
2-(1-methyl-imidazol-4yl)ethylamine, 2-(3-metyl-limidazol-4yl)ethylamine,
β-alanyl-histidine-(carnosine), 7-chloro-4(amino-1-methylbutylamino)-quinoline,

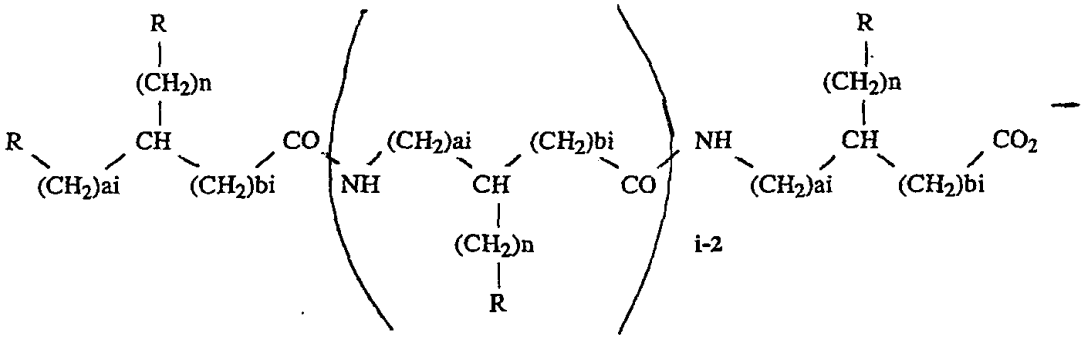
N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine,

8-(4-amino-1-methylbutylamino)-6-methoxy-quinoline (primaquine),

N^4-(6-methoxy-8-quinolinyl)1,4-pentanediamine, quininic acid,

quinoline carboxylic acid, pteroïc acid, nicotinic acid, quinolinic acid.

According to another advantageous embodiment, the oligomeric conjugate of the invention contains an oligomer of the following formula :



wherein

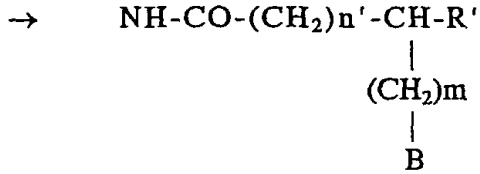
*ai is an integer varying from 0 to 10,

* bi is an integer varying from 0 to 10,

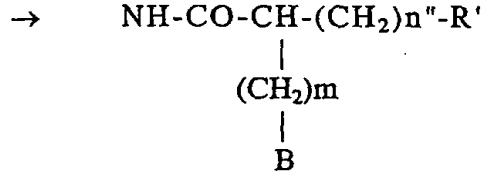
* i = degree of polymerization from 5 to 50, and particularly 10 to 40, and preferably 20,

* n = is an integer varying from 1 to 6, and preferably 4,

* R represents in a ratio of 50 % to 100 % (corresponding to a number u)



or



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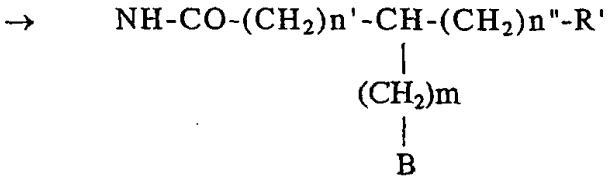


m is an integer varying from 1 to 6,

n' is an integer varying from 0 to 6,

n" is an integer varying from 0 to 6,

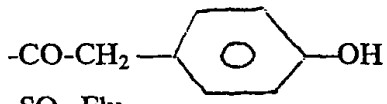
B is a weak base as defined above,

R' represents NH_3^+ (corresponding to a number p),
or NH (corresponding to a number q) substituted by

$-CO-CH_3$

$-CO-(CHOH)rH$ r being from 1 to 15 preferably 2 to 7

$-CO-(CH_2)s-(CHOH)rH$ r being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 preferably 4



$-SO_2-Flu$

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

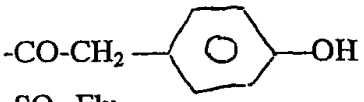
* R represents in a ratio of 0 % to 50 % (corresponding to $f : 0 < f \leq u$)

- NH_3^+ (corresponding to a number j),
- NH (corresponding to a number k), substituted by

$-CO-CH_3$

$-CO-(CHOH)rH$ r being from 1 to 15 preferably 1 to 7

-CO-(CH_2)s-(CHOH)rH r being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 preferably 4

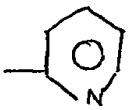


-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

- H (corresponding to a number h)
- (CH_2) nH, n being an integer from 1 to 6 (corresponding to a number h)
- (CH_2) n-OH n being an integer from 1 to 6 (corresponding to a number h)
- (CH_2) n-SA' A' = H , CH_3 or 

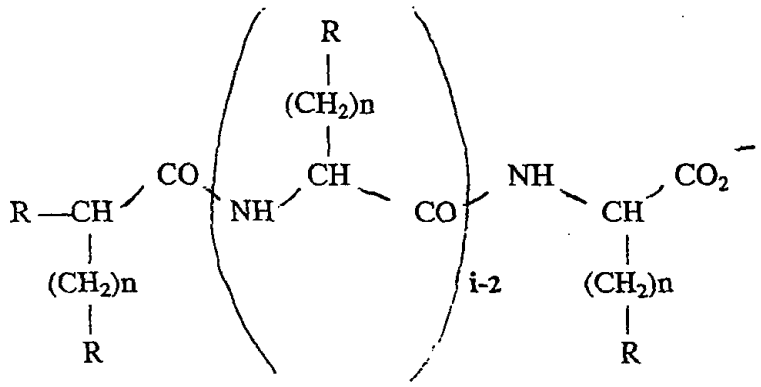
n being integer from 1 to 6 (corresponding to a number h)

with
- i = u + j + k + h
- total number of α NH_3^+ = p = u-q
- total number of ω NH_3^+ = j = f-(k + h)
- total number of NH_3^+ = m = p + j + 1

with the proviso that :

1) $u \geq i/2$
2) $m \geq i/2$

According to another advantageous embodiment, the oligomeric conjugate of the invention contains an oligomer of the following formula :



wherein

* i = degree of polymerization from 5 to 50, and particularly 10 to 40, and preferably 20,

* n = is an integer varying from 1 to 6, and preferably 4,

* R represents in a ratio of 50 % to 100 % (corresponding to u)

→ NH-CO-(CH$_2$)n'-CH-R'
|
(CH$_2$)m
|
B

or

→ NH-CO-CH-(CH$_2$)n"-R'
|
(CH$_2$)m
|
B

or

→ NH-CO-(CH$_2$)n'-CH-(CH$_2$)n"-R'
|
(CH$_2$)m
|
B

m is an integer varying from 1 to 6,

n' is an integer varying from 0 to 6,

n" is an integer varying from 0 to 6,

B is a weak base as defined above,

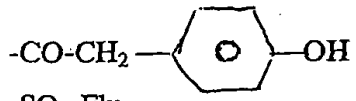
R' represents NH_3^+ (corresponding to a number p),

or NH (corresponding to a number q) substituted by

-CO-CH_3

-CO-(CHOH)rH — r being from 1 to 15 preferably 1 to 7

-CO-(CH_2)s-(CHOH)rH — r being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 and preferably 4



-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

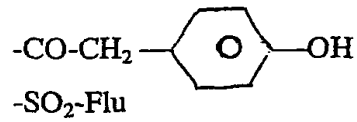
* R represents in a ratio of 0 % to 50 % (corresponding to f : $0 < f \leq 1$)

- NH_3^+ (corresponding to a number j),
- NH (corresponding to a number k), substituted by

-CO-CH_3

-CO-(CHOH)rH — r being from 1 to 15 preferably 1 to 7

-CO-(CH_2)s-(CHOH)rH — r and s being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 and preferably 4



-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

- H (corresponding to a number h)
- $(CH_2)_nH$, n being an integer from 1 to 6 (corresponding to a number h)
- $(CH_2)_n$-OH n being an integer from 1 to 6 (corresponding to a number h)
- $(CH_2)_n$-SA' A' = H , CH_3 or 

n being integer from 1 to- 6 (corresponding to a number h)

with
- i = u + j + k + h
- total number of α NH_3^+ = p = u-q
- total number of ω NH_3^+ = j = f-(k + h)
- total number of NH_3^+ = m = p + j + 1

with the proviso that :

1) $u \geq i/2$
2) $m \geq i/2$.

According to another advantageous embodiment, the oligomeric conjugate of the invention contains an oligomer of the formula above defined, wherein

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0

R' = NH^+_3

m = 1

B = imidazole

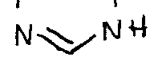
R = NH-CO-CH- NH_3^+

CH_2

(f) R = NH^+_3

u = 12

j = 7



or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'

(CH_2)m

B

wherein

n' = n" = 0

R' = NH^+_3

m = 1

B = imidazole

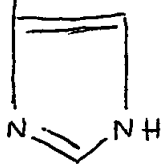
R = NH-CO-CH- NH_3^+

CH_2

(f) R = NH^+_3

u = 16

j = 3

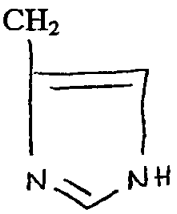


or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2



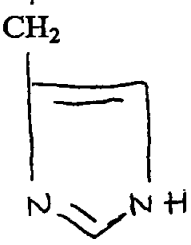
(f) R = NH^+_3
u = 19
j = 0

or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2



(f) R = $CO-CH_3$
u = 11
k = 8

or

i = 19 n = 4 (l) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2

(f) R = $CO-CH_3$
u = 15
k = 4

or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2

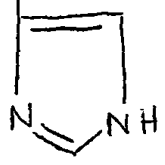
(f) R = $CO-(CHOH)_rH$
r = 5
u = 12
k = 3

or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

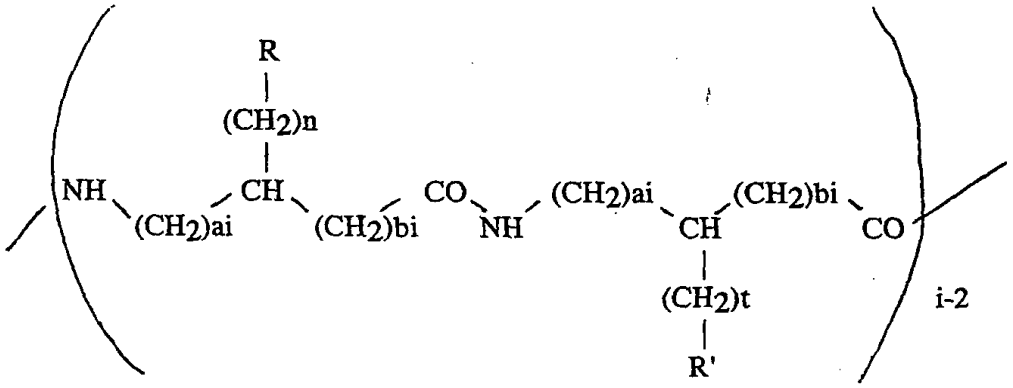
wherein

n' = n" = 0
(q) R' = NH-CO-CH_3
m = 1
B = imidazole
R = NH-CO-CH- $NH-CO-CH_3$
|
CH_2



(f) R = NH^+_3
u = 16
f = 4
k = 3

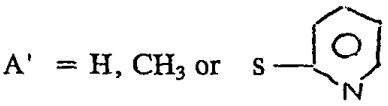
An example of mixed oligomer is the following :



i-2

wherein

n = 4 R' = H, CH_3, $CH(CH_3)_2$, CH_2,-$CH(CH_3)_2$
$CH(CH_3)$-CH_2-CH_3, CH_2OH,
CHOH- CH_3, CH_2-CH_2-S- CH_3
(CH_2)n H, (CH_2)n-OH, (CH_2)n-SA'

A' = H, CH_3 or 

R = NH-CO-(CH_2)n' -CH-(CH_2)n" -R"
|
(CH_2)m
|
B

t varies from 1 to 6

As an example, when, in the above formula n = 4, ai = bi = 0, t = 1, R' = H, the monomers are lysine and valine.

The invention also relates to a composition containing a mixture of at least one oligomeric conjugate as defined above, with at least one biological molecule, such as a peptide, an oligoside or an oligonucleotide derivative, or a mixture thereof.

In the composition of the invention, the oligomeric conjugates can be associated with a biological molecule, in particular an oligoanion, such as an oligonucleotide, an anionic peptide or an anionic oligoside, via electrostatic interactions.

An anionic oligoside can be a sulfated oligoside, succinylated oligoside, phosphorylated oligoside, sialylated oligoside or an oligoside containing pyruvilidenyl groups.

The invention also relates to a combined preparation containing as active substance the following individual components, in the form of a kit-of-parts :

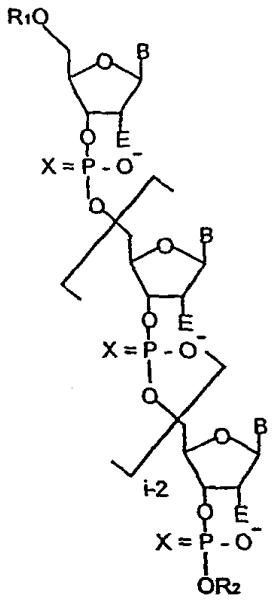
- at least an oligomeric conjugate as defined above,
- at least one biological molecule, such as a peptide, an oligoside or an oligonucleotide, or a mixture thereof,

for the simultaneous, separate or sequential use, for the *in vitro*, the *in vivo* or the *ex vivo* transfer of said biological molecules into the cytosol and/or the cell nucleus.

The invention also relates to a complex between at least one oligoanion which can be an anionic peptide, an anionic oligoside or an oligonucleotide or a mixture thereof, a mixture of at least one non negatively charged biological molecule and of at least one oligoanion, and at least one positively charged oligomeric conjugate as defined above, the association between the oligoanion and the oligomeric conjugate being electrostatic in nature.

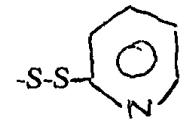
According to another advantageous embodiment, the biological molecule is chosen among oligonucleotides, peptides, oligosaccharides or a mixture thereof.

An oligonucleotide used in the invention can be of the following formula :



wherein *i* varies from 10 to 30, X represents O or S, B is a nucleic base U, A, T, G, C or a modified form such as a biotinyl or fluorescent labelled base which can be in α or β anomeric position, R1 and R2 represent independently from

each other H, OH, (CH_2)n-A, $[(CH_2)_2\text{-}O]_n\text{-}CH_2\text{-}CH_2\text{-}A$, A being H, OH, NH_2, COOH,



, n being an integer from 1 to 6,

E represents H, OH, OCH_3, OCH_2CH_3, $O(CH_2)_2CH_3$, $O(CH_2)_3CH_3$, $O(CH_2)_4CH_3$, $O\text{-}CH_2\text{-}CH_2\text{-}O\text{-}CH_3$

As example of oligonuclotides, one may cite the following :

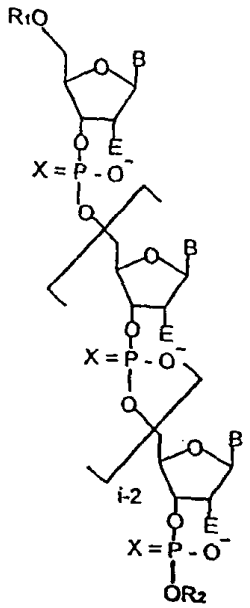
GEM 91
phosphorothioate (X = S) oligonucleotide i = 25
CTC TCG CAC CCA TCT CTC TCC TTC T
complementary to the AUG initation site of gag HIV-1 gene

ISIS 1939
phosphorothioate (X = S) oligonucleotide i = 19
CCC CCA CCA CTT CCC CTC T
complementary to the 3' non coding region of ICAM-1 mRNA.

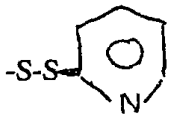
A mixture of an oligonucleotide and a peptide is defined as an oligonucleotide linked to a peptide, and a mixture of an oligonucleotide and an oligoside is defined as an oligonucleotide linked to an oligoside.

A mixture of an oligoside and a peptide is defined as an oligoside linked to a peptide.

A mixture of an oligonucleotide and a peptide or a mixture of an oligonucleotide and an oligoside used in the invention can have the following formulae :



wherein *i* varies from 10 to 30, X represents O or S, B is a nucleic base U, A, T, G, C or a modified form such as a biotinyl or fluorescent labelled base which can be in α or β anomeric position, R1 and R2 represent independently from each other H, OH, (CH_2)n-A, $[(CH_2)_2\text{-}O]_n\text{-}CH_2\text{-}CH_2\text{–}A$, A being H, OH, NH_2, COOH,



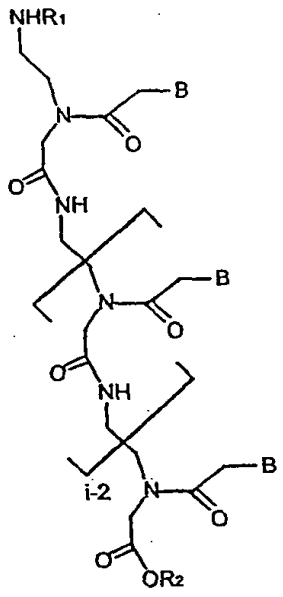
, n being an integer from 1 to 6,

or a peptide or an oligoside,

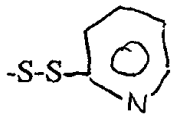
E represents H, OH, OCH_3, OCH_2CH_3, $O(CH_2)_2CH_3$, $O(CH_2)_3CH_3$, $O(CH_2)_4CH_3$, $O\text{-}CH_2\text{-}CH_2\text{-}O\text{-}CH_3$

When R1 and/or R2 represent a peptide, the mixed oligoanion is a peptido-oligonucleotide, and when R1 and/or R2 represent an oligoside, the mixed oligoanion represents a glyco-oligonucleotide.

An example of oligonucleotide is a peptide nucleic acid (PNA) represented by the following formula :



wherein - R1 and R2 represent independently from each other H, OH, (CH_2)n-A, $[(CH_2)_2\text{-}O]_n\text{-}CH_2\text{-}CH_2\text{-}A$, A being H, OH, NH_2, COOH,



, n being an integer from 1 to 6,

- i is an integer varying from 10 to 30.

In the composition of the invention, the oligonucleotide can be simple or double stranded, or can form a triplex (three strands) or a quadruplex (4 strands).

The invention also relates to the use of an oligomer conjugate as defined above, for the intracellular transfer of biological molecules into the cytosol or/and into the cell nucleus *in vitro*, *ex vivo* or *in vivo*.

The invention also relates to the use of an oligomeric conjugate as defined above or of a composition as defined above, or of a combined preparation as defined above, for the intracellular *in vitro*, *ex vivo* or *in vivo* transfer of a peptide, an oligoside or an oligonucleotide, or a mixture thereof, into the cytosol or/and in to the cell nucleus of cells.

The invention also relates to the use of an oligomeric conjugate as defined above or of a composition as defined above, or of a combined preparation as defined above, wherein the cells are chosen among muscular, epithelial, endothelial, or myeloid cells such as monocytes, macrophages, fibroblasts, leukocytes and granulocytes, osteoblasts, as well as dendritic, stem, neuronal, or dermal cells.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of an oligonucleotide, wherein an oligonucleotide and an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is :

- transfer of an antisense oligonucleotide in the cytosol and/or the cell nucleus where it binds and blocks the complementary mRNA sequence,

- transfer of an oligonucleotide as activator into the cytosol where it depresses or activates a second messenger in the cytosol, or the corresponding gene in the nucleus,

- transfer into the cytosol and/or the cell nucleus of oligonucleotides corresponding to a repetitive bacterial type DNA sequence with stimulating or immunodepressive activity.

The transfer of an antisense oligonucleotide in the cytosol where it binds to the complementary mRNA sequence and blocks its traduction leading to inhibition of the synthesis of the gene product, can be carried out as described hereafter in the legends of Figures 1, 2 and 3.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of an oligonucleotide, wherein an oligonucleotide and an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is :

- transfer into the cytosol and/or the cell nucleus of RNA or DNA oligonucleotide acting as decoys which inhibit gene expression by blocking the binding of regulatory factors to the authentic DNA region such as short RNA oligonucleotides corresponding to the HIV-TAR sequence inhibiting HIV expression and replication by blocking the binding of the HIV regulatory protein at Tat to the TAR region,

- transfer into the cytosol and/or the cell nucleus of ribozymes (RNA oligonucleotides) which inhibit gene expression by cleaving the mRNA.

The transfer into the nucleus of an oligonucleotide (triplex forming ODN, TFO) where it binds to target DNA at oligopurine sites where they form a triple-helical structure, leading to the inhibition of the gene expression, can be processed as hereafter described.

As an example of the specific TFO is an oligonucleotide 5'-$A_4GA_4G_6A$-3' directed against the polypurine track (PPT) in the NEF-HIV-1 gene.

The transfer of the oligonucleotide as activator of the immune response can be carried out as follows :

As an example the double strand RNA polyinosinic-polycytidylic acid (poly(I:C) for the increase of the tumoricidal activity of macrophages or for the stimulation of natural killer lymphocyte cytotoxicity.

The transfer of double stranded polynucleotide as activator of the immune response can be illustrated by the following :

Polyinosinic-polycytidylic acid (poly(I:C)) is known to increase the tumoricidal activity of macrophages *via* a TNF-α mediated cytolysis.

Thioglycolate-elicited peritoneal macrophages (2.5×10^5) are plated in 16 mm diameter well multiwell plates in serum free RPMI medium. Upon 2 h incubation at 37°C, non-adherent cells are discarded. Adherent cells are cultured in 0.5 ml of serum free RPMI medium in the absence or the presence of polyI:C and in the absence or the presence of histidylated oligolysine. Culture supernatants are collected after 24 h incubation at 37°C. Before testing, supernatants are rendered cell-free by centrifugation at 2000 g for 10 minutes. The cytotoxic activity of macrophage culture supernatants is determined by using L929 cells pretreated for 2 h at 37°C with 2 μg/ml actinomycin D. Actinomycin D pretreated L929 cells are seeded in 96-wells microplates (4×10^4 cells in 0.05 ml serum free RPMI medium per well). After 3-4 h at 37°C, diluted supernatants from stimulated macrophages are added (0.05 ml) and the cells are incubated at 37°C for 18-20 h. Microplates are washed with PBS and the percentage of cell lysis is determined after staining the cells with 0.05 ml of crystal violet (0.2% in 2 % ethanol). The plates are washed with tap water and the dye is solubilized by adding 0.06 ml per well of 0.3 % sodium dodecyl sulfate. Absorbance of each well is read at 570 nm. The % cytotoxicity is calculated according to $(A_{NS} - A_S)/A_{NS} \times 100$ where A_{NS} and A_S are the absorbances of wells containing target cells incubated with supernatant dilutions of non stimulated and stimulated macrophages, respectively.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of peptide, wherein a peptide and an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is a transfer of said peptide into the cytosol.

The transfer of a peptide can be illustrated by the following :

A) Cells are incubated for 4 h at 37°C with 1 μM fluorescein-labelled peptide (*F-S*-CGEEDTSEKDEL) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

B) Dendritic cells are incubated for 4 h at 37°C with 1 μM c-myc epitope peptide (SMEQKLISEEDLNFELDEA) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde in the presence of 0.5 % saponine, washed and then incubated for 1 h with anti c-myc epitope monoclonal antibody (9E10) in PBS containing containing 10 mg/ml BSA and 0.1% saponin. Cells are washed and further incubated for 1 h in the presence of fluorescein-labelled anti-mouse IgG F(ab)' fragments in PBS containing containing 10 mg/ml BSA and 0.1% saponin. Cells are washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

The fixation of a peptide to intracellular cofactor can be carried as described in the following example:

An oligopeptide corresponding to a prostate specific antigen (PSA) epitope mixed to the oligomeric conjugate is transferred into the cytoplasm of macrophages.

The oligopeptide is fixed there to heat shock protein s (HSP90, HSP70) to form HSP-peptide complexes which are then re-expressed at the surface of macrophages. This complex formed with the HSP cofactor stimulate macrophages and enhance the immune response to the PSA antigen.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of peptide, wherein a peptide and an oligomeric conjugate as defined

above, or a composition as defined above, or a combined preparation as defined above, in particular wherein the peptide is an antigenic peptide, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is a transfer of said antigenic peptide in the cytosol of antigen presenting cells (macrophages, dendritic cells and B cells) where they are processed in proteosomes in order to bind to MHCI molecules, allowing the presentation of the antigenic epitope fixed on MHCI.

In vitro evaluation of antigen presentation can be illustrated by the following:

Dentritic cells were incubated for 4 h at 37°C with the nonadecapeptide (185-203) from the C-terminal part of the HIV-1 Nef protein containing the nonapeptide (190-198) (AFHHVAREL) in the absence or in the presence of an histidylated oligolysine. Cells are washed and further incubated for 24 h at 37°C in the absence of peptide and histidylated oligolysine. MHC class I presentation of peptide antigen was evaluated by Cr^{51} cytotoxic assay by using a CTL clone sensible to the peptide. DCs were labelled with Cr^{51} (target cells : T) and then incubated at 37°C for 4 h in the presence of the CTL clone (effector cells : E) at E/T ratios ranged from 1 to 100. The supernatants are collected and the radioactivity in the supernatant was recorded. The % of specific Cr^{51} release is calculated according to $(A_{NS} - A_S)/A_{NS} \times 100$ where A_{NS} and A_S are the radioactivity in supernatant dilutions of dentritic cells incubated in the absence and the presence of CTL cells, respectively.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of a oligoside, wherein an oligoside and an oligomeric conjugate as defined above, or a composition as defined above, or a combined preparation as defined above, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is a transfer of said oligoside into the cytosol and/or the cell nucleus.

An example of transfer of an oligoside can be illustrated by the following :

Cells are incubated for 4 h at 37°C with 0.5 mg/ml fluoresein-labelled dextrans (either Mw 4000 or Mw 70000) in the absence or in the presence of an oligomeric conjugate. Cells are washed with PBS, fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v)

containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical aperture 1.4).

An example of transfer of a negatively charged oligoside can be illustrated by the following :

Cells are incubated for 4 h at 37°C with 0.5 mg/ml fluoresein-labelled polyanionic dextrans (either Mw 3000 or Mw 70000) in the absence or in the presence of an oligomeric conjugate. Cells are washed with PBS, fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical aperture 1.4).

The fixation of an oligoside to intracellular cofactor can be carried as described in the following example:

An oligoanion with silicated saccharidic components complexed according to the invention is transported into the cytoplasm of human cells where it binds to intracellular cofactors or second messengers such as NF kappa B. This binding causes nuclear transfer of the cofactor which derepress or stimulates genes coding for cytokines (such IL1, TNF-α, IL-12).

This results in a marked stimulation of the human cell cultured in the presence of the complex.

The invention also relates to a pharmaceutical composition, comprising as active substance at least an oligomeric conjugate as defined above, or a composition as defined above, or a combined preparation as defined above, or in association with a pharmaceutically acceptable vehicle.

The invention also relates to the use of an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, or for the preparation of a drug for use in the treatment of cancer, inflammatory or immunology diseases (such as graft rejection, allergy, auto-immunity) or infectious diseases.

The invention also relates to a kit or case containing :

- an oligomeric conjugate as defined above, substituted by a protonable residue leading in a weak acid medium to a destabilization of cellular membranes, this oligomeric conjugate being able to comprise a recognition signal, which is previously fixed or not on the above-said conjugate, said recognition signal being dependent upon the cell to target,
- at least one biological molecule to transfer,
- optionally reagents enabling the possible binding of the recognition signal on the above-said oligomeric conjugate,
- optionally reagents enabling the formation of a composition as defined above, or of a combined preparation as defined above,
- reagents enabling the transfer of the biological molecule in the cytosol and/or the cell nucleus.

LEGEND OF THE FIGURES

Inhibition of luciferase gene expression by GEM-91.

Figure 1 shows the activity of GEM-91, an antisense phosphorothioate oligonucleotide (PS-ODN) (CTC TCG CAC CCA TCT CTC TCC TTC T) complementary to the AUG initiation site of gag HIV-1 gene. The effect of histidylated oligolysines was evaluated by using pRET-Luc cells (a rabbit smooth muscle cell line). These cells produce endogenous luciferase under the control of the human phosphoglycerate kinase promoter and the luciferase gene sequence around the AUG codon was replaced by the initiator AUG codon and several downstream codons of gagHIV-1 gene. The results showed that the activity of GEM-91 (IC_{50} > 5 μM) was increased more than 10 times in the presence of 20 μM HoK2 (IC_{50} 0.25 μM). Whilst, no significant inhibition was obtained in the presence of HoK3 in which the α-NH_2 histidyl residues were acetylated, suggesting that interactions between ODN and histidylated oligolysines were involved. pRET-Luc cells, seeded onto 24-well plates (2 x10^5 cells/well), were treated for 4 h at

37°C in DMEM supplemented with 2 % FBS containing various concentrations of GEM-91, (■) in the absence of histidylated oligolysine, ( ) in the presence of 20 μM HoK2 or (○) in the presence of 20 μM HoK3. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. Then, FBS was raised to 6 % and cells were further incubated for 18 h. Luciferase gene expression was measured by recording luminescence for 4 s. The percentage of luciferase inhibition was calculated by using , [(RLU^{ODN}-RLU)/RLU] x 100 where RLU^{ODN} and RLU were the luciferase activity into cell lysates of cells incubated in the absence and in the presence of ODN, respectively. Results shown typical of experiments carried out in triplicate and repeated at least twice. Data are means ± standard deviation.

Inhibition of TNF-α induced ICAM-1 expression by ISI 1939.

Figure 2 shows the inhibitory effect of TNF-α induced ICAM-1 expression by ISIS 1939 (CCCCCACCACTTCCCCTCT), an antisense phosphorothioate oligonucleotide (PS-ODN) targeted to the 3' non-coding region of ICAM-1 mRNA. The results showed that TNF-α induced ICAM-1 expression was inhibited by ISIS 1939 in the presence of 20 μM of histidylated oligolysines. HoK2 (IC_{50} of 0.25 μM) appeared to be more efficient than HoK1 (IC_{50} of 0.5 μM) probably because HoK2 bore less histidyl residues than HoK1 (15 *versus* 12). The inhibition was very low in the absence of histidylated oligolysines even up to 1 μM ODN (20 % inhibition). A549 cells (ATCC CCL 185, ATCC Rockville, MD) were plated onto 96-wells microtiter plates (10^4 cells /well). The day after, culture medium was removed and cells were washed. Cells were incubated at 37°C for 4 h in 100 μl DMEM serum-free medium containing ISIS 1939 ODN either in the absence (■) or in the presence of 20 μM (●) HoK1 or (□) HoK2. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. One volume of fresh medium containing 10 ng/ml TNF-α was added and cells were further incubated for 18 h. ICAM-1 expression was quantified by ELISA using anti-ICAM-1 antibodies. Cells were washed 3 times with 200 μl of PBS and fixed for 20 min at room temperature in PBS containing 20 mg/ml paraformaldehyde. Then, cells were incubated for 90 min at 37°C with anti-ICAM 1 mouse antibody (Becton

Dickinson) diluted 20 times in PBS containing 20 mg/ml BSA. Cells were washed 3 times with PBS and then incubated for 1 h at 37°C with an anti-mouse horseradish peroxydase conjugate (Becton Dickinson) diluted 2000 times in PBS containing 20 mg/ml BSA. After 3 whashes, the peroxydase activity was assessed by using 100 μl of o-phenylenediamine dihydrochloride peroxydase substrate tablet set (Sigma). After X min incubation at 37°C, the reaction was stopped by adding 25 μl of 3 N H_2SO_4 and the absorbance read at 492 nm. All calculations were made relative to untreated controls in the absence or in the presence of TNF-α. The percentage of TNF-α–induced expression of ICAM-1 was calculated as follows : $[(A_{TNF\text{-}\alpha}{}^{ODN} - A_0) / (A_{TNF\text{-}\alpha}-A_0)] \times 100$ where $A_{TNF\text{-}\alpha}{}^{ODN}$ was the absorbance of ODN treated and cytokine-induced cells, A_0 the absorbance of cells incubated without ODN and TNF-α, and $A_{TNF\text{-}\alpha}$ the absorbance of cytokine-induced cells incubated without ODN. Results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are means ± standard deviation of the percentage of control ICAM-1 expression induced by TNF-α.

Effect of histidylated polymers on the intracellular location of PS-ODN.

Figure 3 shows that histidylated oligolysines induced cytosolic and nuclear delivery of ODN. A549 cells incubated for 4 h at 37°C with 0.125 μM *F*-PS-ODN in the absence of histidylated oligolysine exhibited a faint vesicular staining (Fig 3-a). In the presence of either HoK1 (Fig 3-b) or HoK2 (Fig 3-c), the fluorescent staining was more intense in agreement with the flow cytometry analysis. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. In addition, the vesicles appeared bigger and the cytosol and the nucleus were also labelled while vesicles were smaller and neither the cytosol and the nuclear were staining in the absence of histidylated oligolysine (Fig 3-a). The cytosolic and nuclear staining was greater in the presence of HoK2 than in the presence of HoK1, probably because HoK2 contained more histidyl residues than HoK1 (15 *versus* 12). In contrast, the cell associated fluorescence was low in the presence of HoK3 suggesting that interactions between ODN and HoK1 and HoK2 might favor the ODN uptake (Fig 3-c). Unsubstituted oligolysine had no effect on the ODN uptake and ODN accumulation (Fig 3-d). A549 cells (ATCC CCL 185,

ATCC Rockville, MD) were seeded onto sterile coverslips in 20-mm wells (2 x 10^5 cells/ well) and allowed to adhere. Cells were incubated in the presence of 0.125 μM fluorescein-labelled PS-ODN for 4 h at 37°C (a) in the absence or in the presence of 20 μM (b) HoK1, (c) HoK2, (d) HoK3 or (e) Plk (Oligolysine containing 19 lysyl residues and non substituted by hisditine used as control). Cells were fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells were analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical apErture 1.4).

EXAMPLES

Preparation of histidylated oligolysines :

Oligolysine (Poly-L-lysine, HBr ; average molecular weight of 3950 ; average degree of polymerization of 19) (Bachem Feinchemikalien, Bubendorf, Switzerland) (1 g in 200 ml H_2O) was passed through an anion exchange column (Dowex 2 x 8, ^-OH form, 20-50 mesh) in order to remove bromide ions. The eluate was neutralized with a 10 % *p*-toluene sulfonic acid solution in water and freezed-dried.

Example 1 : Preparation of HoK1

Oligolysine *p*-toluene sulfonate salt (50 mg ; 8.6 μmol) in 2 ml dimethylsulfoxide (Aldrich, Strasbourg, France) in the presence of diisopropylethylamine (50 μl ; 344 μmol) (Aldrich) was reacted for 20 h at 20°C with (Boc)His(Boc)-OH (64 mg ; 146 μmol) (Novabiochem, Bad Soden, Germany) in the presence of benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) (Richelieu Biotechnologies, Saint Hyacinthe, Canada) (159mg ; 358 μmol). The residual ε-amino groups of oligolysine was then substituted with gluconoyl residues (GlcA) : δ-gluconolactone (86mg ; 48 μmol)

(Aldrich) and diisopropylethylamine (134 µl ; 921 µmol) were added and the solution was stirred for 20h at 20°C. The *N*-protecting Boc groups were removed by acidic treatment by adding 10 volumes of a H_2O/trifluoroacetic acid mixture (1:1 ; v/v) for 24h at 20°C. Water and trifluoroacetic acid were removed under reduced pressure. HoK1 was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800g for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800g for 15 min), solubilized in distilled water and freezed-dried. The average number of histidyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy at 300 MHz in D_2O according to : $x = 6.(h_{8.7} / h_{Lys})$. DP, where $h_{8.7}$ was the value of the integration of the signal at 8.7 ppm corresponding to the proton (1H C_{12}) of histidyl residues, h_{Lys} that in the range from 1.3 to 1.9 ppm corresponding to the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of oligolysine. The number of histidyl residues bound per oligolysine molecule was 12. The average number of gluconoyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy from : $x = 3/2.(h_G / h_{Lys})$. DP, where h_G was the value of the integration in the range 3.6 to 3.9 ppm of the 4 protons (1H C_{10}, 1H C_{11} and 2H C_{12}) of gluconoyl residues, h_{Lys} that in the range of 1.3 to 1.9 ppm of the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of pLK. The number of gluconoyl residues bound per oligolysine molecule was 3. The number of free ε-amino groups per oligolysine molecule was 4.

Example 2 : Preparation of HoK2

Oligolysine *p*-toluene sulfonate salt (80 mg ; 13.7 µmol) in 3 ml dimethylsulfoxide in the presence of diisopropylethylamine (90 µl ; 620 µmol) was reacted for 6 h at 20°C with the N-hydroxysuccinimidyl derivative of (Boc)His(Boc)-OH (92 mg ; 204 µmol) (Bachem Feinchemikalien). The residual ε-amino groups of oligolysine was then acetylated (Ac) : acetic anhydride (13 µl ; 109 µmol) (Aldrich) and diisopropylethylamine (5 µl ; 35 µmol) were added and the solution was stirred for 30 min at 20°C. The *N*-protecting Boc groups were removed by acidic treatment by adding 10 volumes of a

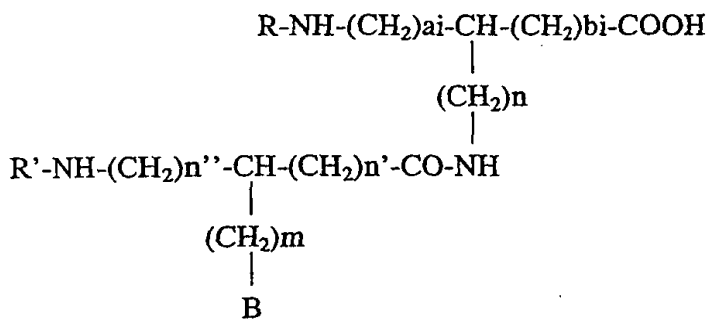
H_2O/trifluoroacetic acid mixture (1 : 1 ; v/v) for 2 h at 20°C. Water and trifluoroacetic acid were removed under reduced pressure. HoK2 was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 g for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800 g for 15 min), solubilized in distilled water and freezed-dried. The average number of histidyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy at 300 MHz in D_2O according to : $x = 6 . (h_{8.7} / h_{Lys})$. DP, where $h_{8.7}$ was the value of the integration of the signal at 8.7 ppm corresponding to the proton (1H C_{12}) of histidyl residues, h_{Lys} that in the range from 1.3 to 1.9 ppm corresponding to the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of oligolysine. The number of histidyl residues bound per oligolysine molecule was 15. The average number of acetyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy from : $x = (h_A / h_{Lys})$. DP, where h_A was the value of the integration at 2.04 ppm of the 3 protons of acetyl residues, h_{Lys} that in the range of 1.3 to 1.9 ppm of the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of pLK. The number of acetyl residues bound per oligolysine molecule was 3. The number of free ε-amino groups per oligolysinemolecule was 1.

Example 3 : Preparation of HoK3

Oligolysine *p*-toluene sulfonate salt (85 mg ; 14.6 μmol) in 3 ml dimethylsulfoxide in the presence of diisopropylethylamine (80 μl ; 555 μmol) was reacted for 20 h at 20°C with N-acetyl-His-OH (288 mg ; 307 μmol) (Sigma) in the presence of BOP (265 mg ; 597 μmol). The residual ε-amino groups of oligolysine was then acetylated (Ac) with acetic anhydride for 30 min at 20°C. HoK3 was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 g for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800 g for 15 min), solubilized in distilled water and freezed-dried. The average number of histidyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy as describe The number of

histidyl residues bound per oligolysine molecule was 15. The number of free ε-amino groups per oligolysine molecule was 1.

Example 4 : Preparation of synthons for synthesis of oligomeric conjugates



wherein

R and R' represent aminoprotecting groups, ai is an integer varying from 0 to 6, bi is an integer varying from 0 to 6, n is an integer varying from 1 to 6, n' is an integer varying from 0 to 6, n" is an integer varying from 0 to 6, m is an integer varying from 1 to 6.

An example of synthon : the Lys(His) synthon

wherein

ai = bi = 0
n = 4
R1 = Fmoc
n' = n'' = 0
m = 1
R' = Boc
B = (NBoc)Imidazole

The N-hydroxysuccinimidyl derivative of $(Boc)_2$-His-OH [$(Boc)_2$-His-OSu] (1g ; 2.2 mmol) is coupled to Fmoc-Lys-OH (722 mg ; 2.2 mmol) for 24 h at 20°C in dimethylformamide (ml). The Lys(His) synthon is precipitated with isopropanol, collected by centrifugation, washed with ether and dried under vaccum. The Lys(His) synthon is purified by cristallization.

Example 5 :

Preparation of an histidylated oligolysine (HoK) containing 20 lysine residues and 20 histidyl residues.

A HoK containing exactly 20 lysyl residues and 20 histidyl residues can be entirely synthetised by using the above Lys (His) synthon. Briefly, 20 Lys(His) synthon are successively assembled on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons are coupled by the HBTU activation method. HoK aree cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. Crude HoK is precipitated with isopropanol and collected by centrifugation. HoK is washed three times with isopropanol, resuspended in distilled water and freezed-dried.

Example 6

Preparation of an oligomeric conjugate containing 17 lysyl residues substituted with 17 histidyl residues and 3 leucyl residues inserted any where in the lys (His) sequence

Oligomers made of exactly 17 lysyl residues substituted with 17 histidyl residues and 3 leucyl residues insert anywhere in the Lys(His) sequence can be entirely synthetised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu were coupled by the HBTU activation method. Oligomers are cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. Oligomers are precipitated with isopropanol and collected by centrifugation. Oligomers are washed three times with isopropanol, resuspended in distilled water and freezed-dried.

Example 7 : Preparation of $(K(His)-KL(His)-L)_7$

An oligomer $(K(His)-K(His)-L)_7$ can be entirely synthetised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu are coupled by the HBTU activation method. The oligomer $(K(His)-K(His)-L)_7$ is cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. The polymer is precipitated with isopropanol and collected by centrifugation. The oligomer is washed three times with isopropanol, resuspended in distilled water and freezed-dried.

~~Example 8 : Preparation of $(K(His)-L-K(His))_7$~~

A oligomer $(K(His)-L-K(His))_7$ can be entirely synthetised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu are coupled by the HBTU activation method. The oligomer is cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. The oligomer is precipitated with isopropanol and collected by centrifugation. The oligomer is washed three times with isopropanol, resuspended in distilled water and freezed-dried.

Table I shows that oligolysine having a DP of either 190, 72 or 36 substituted with less that 45% of histidyl residues, do not allow the transfer of small nucleic acid, particularly oligonucleotides. Indeed, it is necessary to adapt the size of histidylated oligolysines. Conversely, small histidylated oligolysines (DP of 36 or 19) substituted by more than 50% of histidyl residues do not allow efficient gene transfer by histidylated oligolysine/plasmid complexes (Table I). In addition oligolysines substituted with less than 50% histidyl residues are cytotoxic.

Table I : comparative evaluation of gene transfer and oligonucleotide transfer by using histidylated polylysine.

DP	His (%)	DNA	ODN	Cytotoxicity (%)
190	35	100	0	24
190	45	110	0	21
72	23	87-96	0	24
36	22	61-100	0	25
36	53	0-10	20	4
19	25	15-26	0	49
19	45	9	0	40
19	60	nd	100	26
19	80	nd	100	0
19	100	nd	100	0

DP is the oligolysine degree of polymerization. DNA corresponds to transfection by using histidylated oligolysine/pCMVLUC. The transfection efficiency is scored on a 0 to 100 scale. The transfection efficiency is determined from the luciferase activity in cells measured by luminescence. ODN corresponds to cytosolic and nuclear transfer of fluorescein-labelled oligonucleotide in the presence of histidylated oligolysine, evaluated under confocal microscope. Cytotoxicity was evaluated by using the colorimetric MTT assay. (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)